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TITLE: A Novel siRNA-based Approach to Study Mechanisms of Resistance/Action of a New Drug in Treatment of Breast Cancer

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14. ABSTRACT The mechanism by which ET-743 (Yondelis TM , trabectedin) exerts its antitumor activity is not understood. The goal of this study was to study the mechanism of action/resistance of ET-743 in breast cancer cells using a novel siRNA-based approach. Two primers representing the sense and anti-sense DNA template of the random siRNA inserts were cloned into the linearized pFIV-H1/U6 siRNA expression vector. Out of 33 siRNA sequences obtained from vector transformed E.coli colonies, predicted that 67% of the siRNA template sequences would generate functional siRNA sequences and that 82% of the functional siRNA sequences were random. A pool of plasmids encoding the random siRNAs (with a possible 105 to 106 different siRNAs), either transduced with pseudo- lentiviral particles or transfected with electroporation, were tested in the MCF-7 breast cancer cell line. The cell line was then treated with a lethal dose of ET-743 and cytarabine, however no resistant colonies were obtained. Further scaling-up is required to fulfill the goals of this project.					
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INTRODUCTION

ET-743 is an extremely potent cytotoxic agent derived from a marine tunicate. The phase II clinical trials of ET-743 in breast cancer and ovarian cancer patients have demonstrated a promising potential of this novel anti-tumor agent. The exact mechanism by which ET-743 exerts its anti-tumor activity is not known.

The goal of this proposal was to study the mechanisms of resistance/action of ET-743 in breast cancer cell lines using a novel siRNA-based approach and to identify specific genes involved in resistance/action of ET-743.

SPECIFIC AIMS-

1. To construct a siRNA library expressed from two opposing promoters, H1 and U6, in a lentiviral vector,
2. To test the ability of the generated siRNA library to detect drug resistance genes by using two drugs with known mechanisms of resistance, methotrexate (MTX) and cytarabine, using a clonogenic assay and MCF-7 breast cancer cells.
3. To employ the lentiviral siRNA library in a breast cancer cell line, MCF-7, to discover the mechanism(s) of action and/or resistance by selecting ET-743 resistant clones and identifying genes targeted by siRNAs in each clone.

This approach, if successful, might be used as a general strategy to pinpoint resistance/sensitivity mechanisms to any new drug. Understanding how ET-743 causes its effects might enhance our ability to predict response of patients with breast cancer to this agent.

BODY

Specific Aim 1. To construct a siRNA library expressed from two opposing promoters, H1 and U6, in a lentiviral vector,

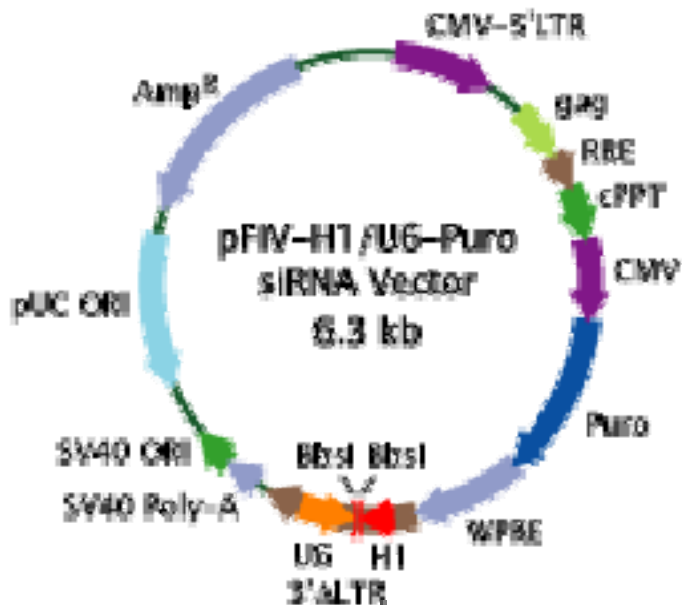


Fig: 1

Two primers representing the sense and anti-sense DNA template of the random siRNA inserts were obtained from Integrated DNA Technologies (Coralville, IA). The primers each contained a fixed 5' sequence of four bases and 25 random bases, possible 4^{25} or approximately 10^{14} siRNA molecules. The sequences of each primer were as follows: Random sense siRNA oligo: 5' AAAGN₂₅ 3' and random anti-sense siRNA oligo: 5' -AAAAN₂₅ -3'. The primers were annealed, phosphorylated and ligated into the linearized pFIV-H1/U6 siRNA expression vector (System Biosciences, Mountain View, CA).

The four fixed bases were complementary to TTTT overhangs on the linearized plasmid, facilitating cloning of the siRNA template sequences. The expression vector contained opposing RNA pol III promoters that were constitutively expressed, and transcription was terminated with TTTT sequences that flank each side of the random N₂₅ sequences. For each ligation reaction, 50 ng of plasmid was used. This represents 6.6×10^8 molecules of plasmid DNA, representing a maximum of 6.6×10^8 possible different siRNA expression plasmids per ligation reaction. DH5 α competent cells (Invitrogen, Carlsbad, CA) were transformed with the ligation reaction. The transformation efficiency of these cells was greater than 1×10^8 transformants per μ g of DNA in the reaction. For each transformation reaction, we used 10 ng of DNA, representing a potential of 1.5×10^6 siRNA expression constructs.

To confirm the presence of potentially functional siRNA sequences and the randomness of the siRNA sequences obtained, colonies were screened by PCR. Primers corresponding to vector sequences flanking the siRNA insert were used to obtain a 170 bp PCR product. The H1 and U6 PCR primers (sequences obtained from System BioSciences, Mountain View, CA) were used. The H1 PCR primer sequence was 5'-CTGGGAAATCACCATAAACGTGAA-3' and the U6 PCR primer sequence was 5'-GCTTACCGTAACTTGAAAGTATTTTCG-3'. However, sequencing of the PCR products yielded poor sequencing data. Plasmid DNA was then isolated from a total of 40 colonies, and clear sequencing data was obtained from 32 samples. For sequencing data, primers further away from the siRNA template were utilized. The sense primer sequence was 5'-TTGCATGTCGCTATGTGTTCT-3' and the anti-sense primer used was 5'-CCCATGATTCCTTCATATTTGC-3'.

The sequences of the 32 siRNA templates were analyzed to predict if they were capable of acting as functional siRNA molecules and to determine the randomness of the siRNA sequences produced. Sequences were only accepted if the sequence flanking the siRNA template aligned exactly with the plasmid and contained RNA termination signals (4-6Ts). Predicted siRNA sequences also contained a random oligo length of 19-29bp, a GC content of 40-60%, and did not contain any stretches of 4 or more of the same base. Sequencing results showed that 15 sequences out of the predicted 19 functional siRNA had unique sequences. There were 4 identical siRNA sequences in the same group of 19 sequences. A nucleotide BLAST search showed that 4 expressed sequences had a match at 15 or more contiguous bases. Six genes total were matched in the nucleotide BLAST search. Given 4 matches out of 33, an estimate of the actual probability of a match was $4/33 = 0.1212$, and a 95% confidence interval was (0.04816, 0.27326) using the Wilson method. Based on this information, one can be fairly certain that the match probability is greater than 0.04816. Since one may reasonably expect that 25% of matches will actually be effective at inhibiting gene activity, a good estimate of the probability of gene inhibition is $0.1212 \times 0.25 = 0.03025$, and it is most likely greater than $0.04816 \times 0.25 = 0.01204$.

Specific Aim 2. To test the ability of the generated siRNA library by using two drugs with known mechanisms of resistance, methotrexate (MTX) and cytarabine, using a clonogenic assay and MCF-7 breast cancer cells.

Four hours after triple transfection :1.5 μ g of random siRNA/pFIV expression plasmid and 3.0 μ g of a packaging plasmid mix combination of pFIV-34N and pVSV-G plasmids (System Biosciences), of the 292T/17 cell

line, the transfection media was removed and complete media was added to each plate. At 36 hours post-transfection, the media was replaced with 7.5ml of media containing 3% FBS. Media containing siRNA library pseudovirus was then collected every 12 hours for 36 hours. At each collection, 7.5ml of fresh media was added to each plate. The collected media was centrifuged at 3,000 rpm for 5 minutes to pellet any cells. In addition, the supernatant was filtered through a 0.45 um nylon filter to remove any remaining cells. Media was collected and filtered at 12, 24, and 36 hours, was pooled, aliquoted, and frozen at -80°C until transductions were performed. Lentiviral vector titers were defined using the Lentiviral Rapid Titer PCR kit (System Biosciences) according to manufacturers protocol, and vector titers were 10^4 to 10^5 iu/ml.

MCF-7 cells were seeded in twenty 10-cm dishes at a density of 2×10^6 cells/plate (total cell number 40×10^6 cells). To transduce cells, 5 ml of pseudoviral supernatant containing 4ug/ml polybrene (Sigma) was added to each plate. As a negative control, 5 ml of media containing 3% FBS was added to an untransduced sample. MCF-7 cells were transduced three times, and then allowed to recover in media containing 3% FBS for 24 hours. All of the cells were then replated in 6-well plates at 2×10^5 cells/well. Control lentiviral vector with copGFP used for vector production and MCF-7 transduction, 3 cycles of transduction provided a transduction efficiency rate of 37%. Cells transduced with siRNA library were treated 24 hours later with ET-743, methotrexate (MTX) and cytarabine (Ara-C) to select resistant cells. Selection media contained drug doses that were 100% lethal for parental MCF-7 cell line after 10-14 days of exposure.

There were no colonies after selection with either ET-743 or anti-metabolites, while puromycin selection gave distinctive colonies. DNA was isolated from both puromycin selected and non-selected transduced MCF-7 cells, and un-transduced MCF-7 cells, and gene existence was checked with PCR by using puromycin N-acetyltransferase (Pac) gene primers (Forward primer: 5' GTA CAA GCC CAC GGT GCG-3' and Reverse primer: 5' AGG TCT CCA GGA AGG CGG-3').

Detection of the Pac gene by PCR.

Expected PCR product of 480bp

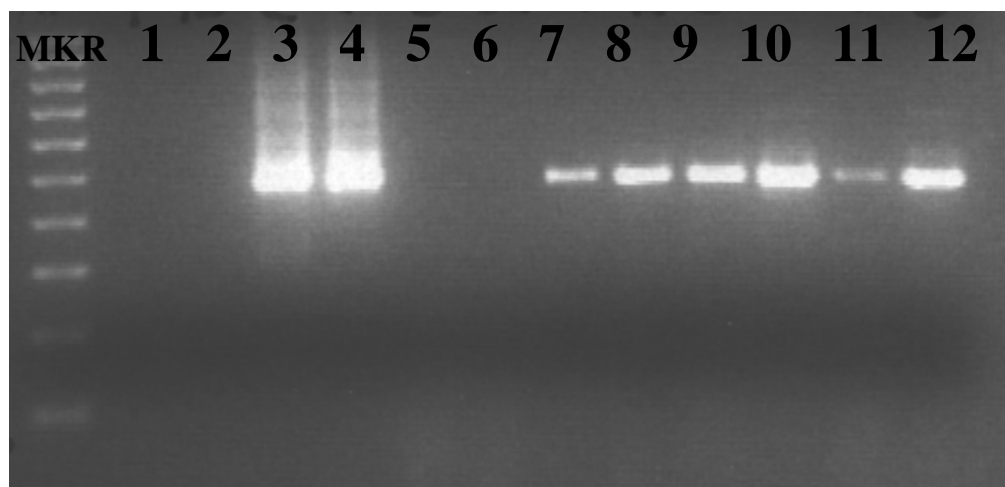


Fig: 2

1-2	Negative control
3-4	Positive control
5-6	Untransduced
7-10	Transduced/Puro Selected
11-12	Transduced Non-Selected

These data suggested that the vector production and/or transduction with the lentiviral vector might limit the number of siRNA's that were efficiently transduced into MCF-7. Therefore we decided to transfer the library with non-viral gene transfer methods, i.e, electroporation.

The MCF-7 cell line was transfected with Amaxa Nucleofector Solutions V (Amaxa Inc.,Gaithersburg, MD) using the manufacturers protocol. For optimizing the protocol, we used SFG^{mpsv}-F/S-DHFR-CD-IRES-eGFP, pmax-GFP (Amaxa Inc) vectors, linearized or circular. For optimization, different doses of plasmid DNA (10 to 15 µg) and different numbers of cells (2 to 8x10⁶ cells) were tested. Cells were passaged without selection for up to 30 days, and GFP expression was checked every other day. None of the conditions provided us expression levels higher than 5% after 4 weeks, and we concluded this method of transfection would not give us reliable, stable gene transfer which would allow us to select resistant cell line clones.

MCF-7 GFP Amaxa Nucleofections

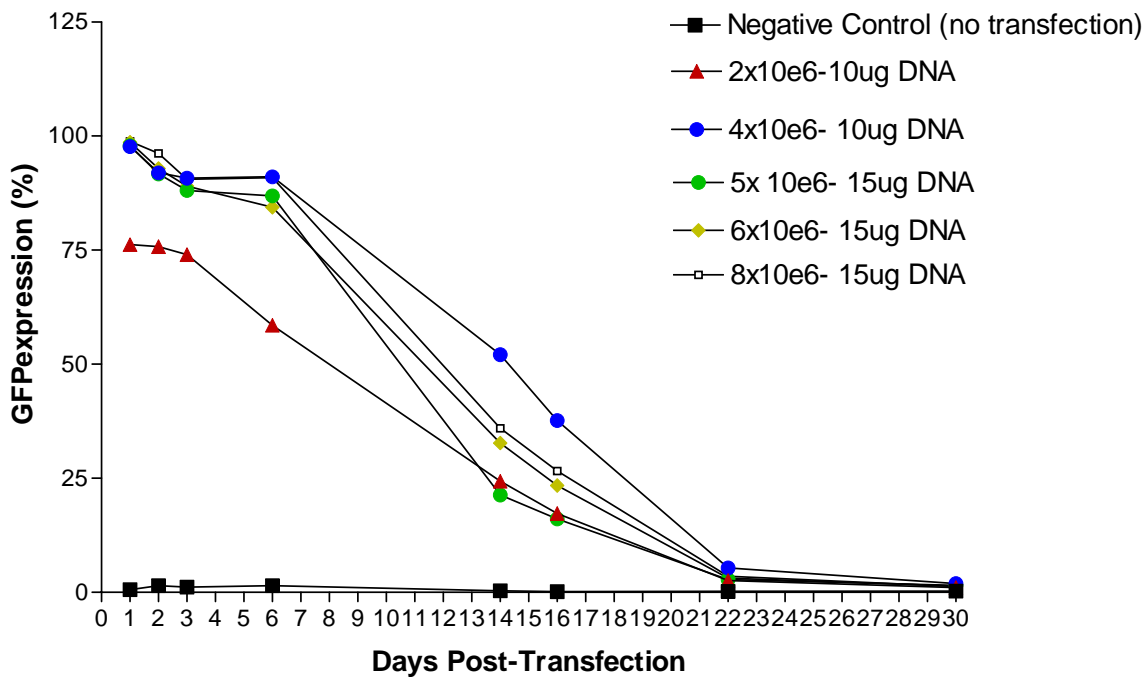


Fig: 3

We concluded that establishment of a sufficiently diverse siRNA library, and the generation of a high titer lentiviral vector supernatant, would require logarithmic scale-up of all steps. Unfortunately a request for additional funds from the DOD to complete this study was not approved.

Key Research Establishments-

1. We successfully generated a siRNA library starting from random primers cloned into the pFIV-H1/U6 expression vector, with a diversity of 1.5×10^6 different siRNA molecules. The results obtained predict that 67% of the siRNA template sequences would generate functional siRNA sequences and that 82% of the functional siRNA sequences were random.
2. We successfully transduced MCF-7 cells with lentiviral vector supernatant, and generated puromycin resistant clones. However, the diversity of the siRNA library was not enough to down-regulate known MTX and ARA-C resistance related genes, approximately 0.067% of the coded genome. We validated the diversity of the siRNA library after lentiviral vector cloning. Sequencing of random E.coli clones indicated that we could have a library with a diversity of 45,454 functional siRNA molecules. However, vector production and target cell transduction steps might have limited the capacity of the siRNA library. The assumption was that all of the lentiviral vector transformed bacteria would have equal chance of exponential growth in LB media, and after plasmid purification representation of each siRNA molecule will be similar to the original pool. Additionally, molecules should have a similar probability of being represented in 292T cell line transfection and then after entiviral vector production. We did not validate those steps with known mixtures of plasmids and testing through the lentiviral transduction and target cell

transduction steps. For a scale-up of siRNA production those steps and these assumptions should also be validated.

For this project our initial suggestion was that after insertion of siRNA containing vector into the genome, the expression and functionality of siRNA molecules would not be influenced by the factors related to insertion site or cell cloning. However, there have been suggestions that expression and stability of the inserted vector could be influenced by the copy number/per cell and the insertion site.

We tested the efficiency of lentiviral transduction for down-regulating targeted genes by using two different chemically synthesized siRNA molecules defined for down-regulating the retinoblastoma gene (first template sequence for siRNA was 5'- aaagGAT ACC AGA T CA TGT CAG A -3', 3'- CTA TGG T CT AGT ACA GTC Taaaa -5' and the second: 5'- aaagCCC AGC AGT TCG ATA TCT A-3', 3'- GGG TCG TCA AGC TAT AGA Taaaa- 5'). We similarly cloned those molecules into the pFIV-H1/U6 siRNA expression vector, generated lentiviral vector supernatant, and transduced H865 small cell lung carcinoma cells with the functional RB gene. With puromycin selection, we obtained 17 clones; all of these clones were PCR positive for the Pac gene, WPRE, and U6-H1 promoter. However only two clones had complete Rb mRNA and protein knock-down. Therefore we conclude that the Integration site of the lentiviral vector could affect the expression level of siRNA, and may be a reason for failer to see down-regulation of known drug resistance genes with our siRNA library.

Reportable Outcome-

1. Zhen Hu, Jennifer Krouse, Debabrata Banerjee, Tulin Budak-Alpdogan and Joseph R. Bertino. A novel siRNA based approach to study mechanisms of resistance/action of a new drug in treatment of breast cancer. ERA of Hope, Philadelphia, June 2005. Poster number 66.

Conclusion-

With the availability of siRNA libraries, with known diversity from commercial sources, this alternative approach is worth exploring for the purpose of discovery of mechanism of action and /or resistance mechanisms of cancer drugs.

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